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Inhibition of cytochrome *c* oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro

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Abstract

L-2-Hydroxyglutaric (LGA) and D-2-hydroxyglutaric (DGA) acids are the characteristic metabolites accumulating in the neurometabolic disorders known as L-2-hydroxyglutaric aciduria and D-2-hydroxyglutaric aciduria, respectively. Although these disorders are predominantly characterized by severe neurological symptoms, the neurotoxic mechanisms of brain damage are virtually unknown. In this study we have evaluated the role of LGA and DGA at concentrations ranging from 0.01 to 5.0 mM on various parameters of energy metabolism in cerebral cortex slices and homogenates of 30-day-old Wistar rats, namely glucose uptake, CO_2 production and the respiratory chain enzyme activities of complexes I to IV. DGA significantly decreased glucose utilization (2.5 and 5.0 mM) by brain homogenates and CO_2 production (5 mM) by brain homogenates and slices, whereas LGA had no effect on either measurement. Furthermore, DGA significantly inhibited cytochrome *c* oxidase activity (complex IV) (EC 1.9.3.1) in a dose-dependent manner (35–95%) at doses as low as 0.5 mM, without compromising the other respiratory chain enzyme activities. In contrast, LGA did not interfere with these activities. Our results suggest that the strong inhibition of cytochrome *c* oxidase activity by increased levels of DGA could be related to the neurodegeneration of patients affected by D-2-hydroxyglutaric aciduria.

Keywords: L-2-Hydroxyglutaric acid; D-2-Hydroxyglutaric acid; Brain metabolism; Cytochrome *c* oxidase

1. Introduction

L-2-Hydroxyglutaric aciduria (LHGA) and D-2-hydroxyglutaric aciduria (DHGA) are rare neurometabolic disorders biochemically characterized by tissue accumulation and high urinary excretion of L-2-hydroxyglutaric acid (LGA) and D-2-hydroxyglutaric acid (DGA), respectively [1–6].

LHGA was first described in 1980 by Duran and colleagues [2] and has now been described in at least 50 patients. Patients with LHGA usually present

Abbreviations: LHGA, L-2-hydroxyglutaric aciduria; DHGA, D-2-hydroxyglutaric aciduria; LGA, L-2-hydroxyglutaric acid; DGA, D-2-hydroxyglutaric acid; MRI, magnetic resonance imaging; ETP, electron transfer flavoprotein; COX, cytochrome *c* oxidase; KRB, Krebs–Ringer bicarbonate buffer; HMVA, D-2-hydroxy-3-methylvaleric acid

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with mental deterioration, seizures, pyramidal and extrapyramidal symptoms and severe cerebellar dysfunction. Ataxia, tremor and choreiform movements, as well as speech delay in infancy or childhood have also been reported [4,7,8]. Although in most patients symptoms presentation occurs in early to middle childhood, a more severe form has been reported in an infant which presented with hypotonia, apnoea and convulsions shortly after birth [9]. An adult form affecting older patients has also been reported [10]. Neuroimaging reveals cerebellar atrophy, progressive loss of myelinated arcuate fibres and alterations in the basal ganglia. Besides the characteristic high levels of LGA in plasma, CSF and urine, a few affected patients present increased concentrations of lactate or Krebs cycle intermediates in CSF, plasma or urine [4,9,11].

DHGA was first recognized by Chalmers and coworkers in 1980, and has now been reported in at least 25 patients [1,6]. It is clinically characterized by at least two variants, a severe form with early-infantile-onset encephalopathy, whose patients usually present with seizures, hypotonia and poor development, as well as enlarged frontal subarachnoid spaces and subdural effusions and signs of cerebral delayed maturation with subependymal cysts in the caudate nucleus detected by MRI. The other variant is clinically milder and more variable, presenting usually with mental retardation, macrocephaly and hypotonia. Delayed cerebral maturation, ventriculomegaly and subependymal cysts are also observed by MRI. Biochemically, besides the high excretion of DGA, lactate and Krebs cycle intermediates are also found in elevated amounts in the urine of a considerable number of patients [5,6]. High excretion of DGA also occurs in multiple acyl-CoA dehydrogenase deficiency (glutaric acidemia type II), which is due to a defect of the electron transfer flavoprotein (ETP) or of the mitochondrial enzyme ETF-ubiquinone oxidoreductase [12].

Despite the intensive clinical investigation and the large number of loading and fast tests, the underlying biochemical defect of these disorders and the origin of DGA and LGA have remained an enigma. Initially L-2-hydroxyglutaric acid dehydrogenase and D-2-hydroxyglutaric acid dehydrogenase deficiencies were considered as potential causes of these diseases. However, the activities of the enzymes were normal or even increased in the liver of affected patients, suggesting that their accumulation may result from a secondary pathway, rather than from the primary substrate of the missing enzyme activity [3,4,13]. Likewise, the effects of these acids on cell metabolism are virtually unknown and await investigation.

Moreover, virtually nothing is known about the pathophysiology of the neurological dysfunction of DHGA and LHGA. However, considering that elevated levels of lactate and/or Krebs cycle intermediates are observed in some patients affected by these disorders, it is conceivable that a primary or functional mitochondrial defect or dysfunction may be associated with these diseases.Therefore, in the present study we investigated the in vitro effects of DGA and LGA on various parameters of energy metabolism such as glucose utilization and CO_2 formation and on the enzyme activities of the respiratory chain complexes in cerebral cortex of young rats in the hope to determine whether the acids could compromise energy production in the brain. Skeletal muscle from human beings were also used in some experiments since, similarly to brain, this tissue has a high amount of mitochondria and thus a high demand of energy.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for the radiolabeled compounds [U-^{14}C]acetate and [U-^{14}C]citrate which were purchased from Amersham International plc, UK.

2.2. Subjects

Thirty-day-old Wistar rats bred in our laboratory were used. Rats had free access to a 20% protein commercial chow and water and were kept in a room with a 12:12-h light/dark cycle and temperature of 24 ± 1°C. Animals were killed by decapitation without anesthesia, the brains were immediately removed and the cerebral cortex was dissected onto an ice-cold glass plate. The experimental protocol was approved by the Ethics Committee for animal re-

search of the Federal University of Rio Grande do Sul, Porto Alegre.

Skeletal muscle specimens obtained from normal human individuals were also used for cytochrome *c* oxidase activity determination. All subjects gave written informed consent to participate in the study. The research was approved by the Committee for Human Research of Hospital de Clínicas de Porto Alegre.

2.3. Tissue preparation

2.3.1. Cerebral cortex slice preparation

Brain cortex was cut to produce 400-μm wide slices using a McIlwain chopper. Slices were pooled, weighed and used for the glucose uptake and CO_2 production assays.

2.3.2. Homogenate preparation

Rat cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.0 (glucose uptake) or pH 7.4 (CO_2 production), or in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI ml^{-1} heparin) (respiratory chain enzyme activities). The homogenates were centrifuged at $800 \times g$ for 10 min and the supernatants kept at −70°C until used for enzyme activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

Human skeletal muscle was homogenized in the same way and the supernatants were used for the determination of cytochrome *c* oxidase (COX) activity. The protein concentration in the supernatants (brain and skeletal muscle) varied from 1.0 to 2.0 mg protein ml^{-1}.

2.4. Glucose utilization

Cerebral cortex slices (100 mg) or a corresponding volume of homogenates (1:10, w/v) prepared in Krebs–Ringer bicarbonate buffer, pH 7.0 (KRB) from overnight-fasted animals were incubated in the same buffer (in a total volume of 1 ml), containing 5.0 mM glucose and DGA or LGA (1–5 mM) in a O_2/CO_2 (19:1) mixture in a metabolic shaker at 37°C for 60 min. Flasks were firstly pre-incubated for 15 min (90 oscillations min^{-1}) in the absence of glucose. Glucose was then added at the beginning of incubation. Control experiments did not contain the acids in the incubation medium [14]. Glucose was measured by the glucose oxidase method [15] and the uptake determined by subtracting the amount after incubation from the total amount measured before incubation.

2.5. CO_2 production

Cerebral cortex slices (50 mg) or a corresponding volume of homogenates (1:10, w/v) were added to small flasks (11 cm^3) containing 0.5 ml Krebs–Ringer bicarbonate buffer, pH 7.4. Flasks were pre-incubated in a metabolic shaker at 37°C for 15 min (90 oscillations min^{-1}). After pre-incubation, 0.2 μCi [U-^{14}C]acetate and 0.5 mM of the unlabeled acetate were added to the incubation medium. In some experiments, 0.1 μCi [U-^{14}C]citrate and 0.5 mM of the unlabeled substrate were used. DGA or LGA (buffered to pH 7.4) was added to the incubation medium at final concentrations of 1.0, 2.5 or 5.0 mM. The controls did not contain the acids. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 65 mm/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation at 37°C, 0.1 ml of 50% trichloroacetic acid was added to the medium and 0.1 ml of benzethonium hydroxide was added to the center wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was measured [16].

2.6. Respiratory chain enzyme activities

The activities of citrate synthase (EC 4.1.3.7) and of the respiratory chain enzyme complexes succinate-DCIP-oxidoreductase (complex II) (EC 1.3.5.1) and succinate:cytochrome *c* oxidoreductase (complex II+CoQ+complex III) (EC 1.10.2.2) were determined in brain cortex homogenates according to the method of Fischer et al. [17]. The activity of cytochrome *c* oxidase (complex IV) (COX) (EC 1.9.3.1) was measured according to Rustin et al. [18], whereas those

of NADH:cytochrome *c* oxidoreductase (complex I+CoQ+complex III) (EC 1.6.5.3) and ubiquinol cytochrome *c* oxidoreductase (complex III) (EC 1.10.2.2) were assayed according to the method described by Schapira et al. [19]. Experimental groups contained various concentrations of LGA or DGA (1, 2.5 and 5 mM). Control groups did not contain any acid in the incubation medium.

2.7. Protein determination

Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as standard.

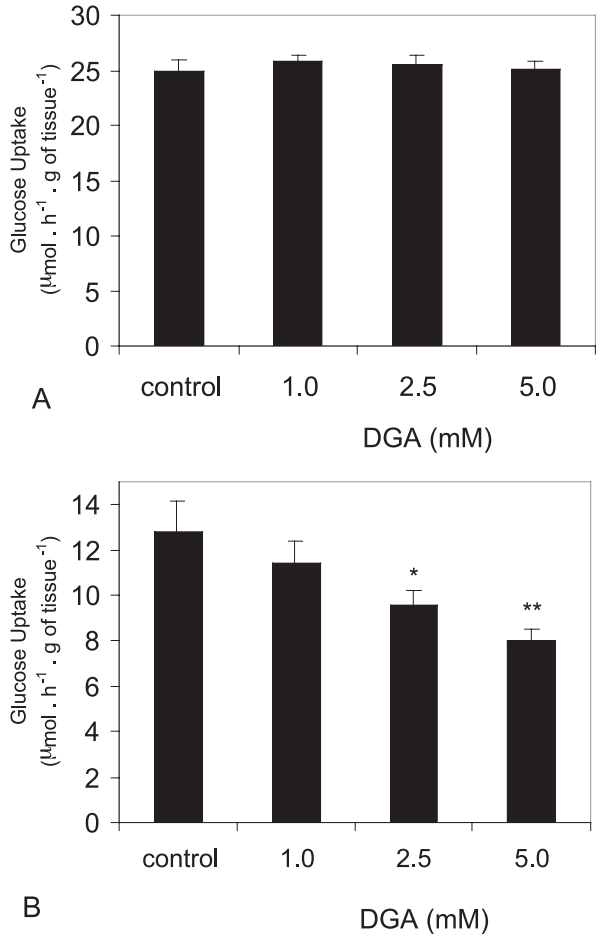

Fig. 1. Effect of D-2-hydroxyglutaric acid (DGA) on in vitro glucose uptake (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex of 30-day-old rats. Values are mean ± S.E.M. for $n = 4$–7 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to controls (Duncan multiple range test). (A) Slices; (B) homogenates.

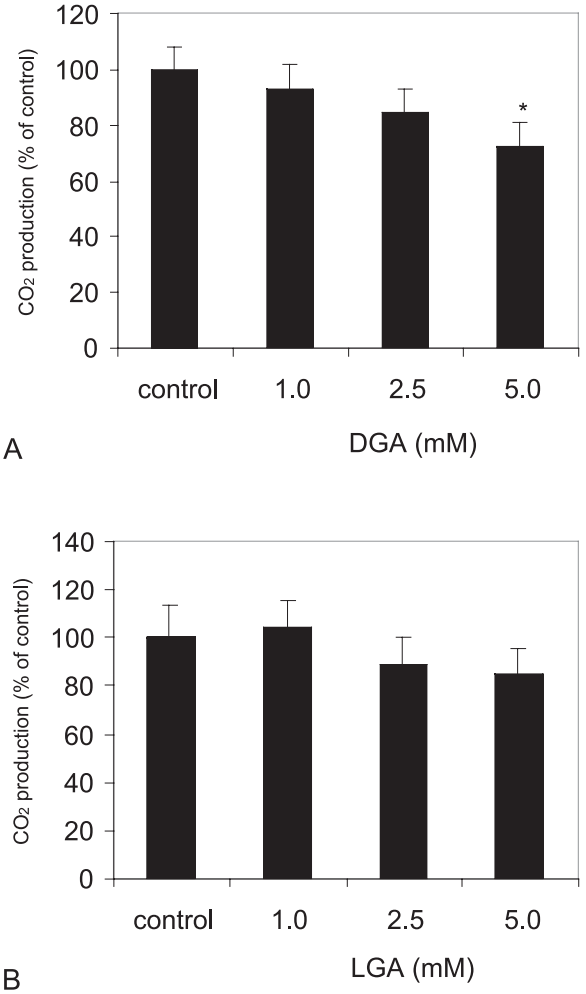

Fig. 2. Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on in vitro CO2 production from [U-^{14}C]acetate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex homogenates of 30-day-old rats. Values are mean ± S.E.M. for $n = 4$–7 per group, and are expressed as percentage of controls. $^{*}P < 0.05$, compared to controls (Duncan multiple range test).

2.8. Statistical analysis

Unless otherwise stated, results are presented as means ± standard error of the mean. Data concerning glucose utilization, CO_2 production and the activities of the respiratory chain enzyme complexes were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. The Student t-test for paired samples was also used for comparison of two means. Differences between the groups were rated significant at a probability error of less than 0.05.

3. Results

We first determined the effect of DGA (Fig. 1) and LGA (Fig. 2), at concentrations varying from 1 to 5 mM, on glucose utilization by cerebral cortex slices and homogenates of 30-day-old rats. DGA at concentrations of 2.5 mM and higher significantly reduced the utilization of glucose by the cerebral homogenates ($F(3,14) = 5.5663$; $P = 0.0143$), but not by the cerebral cortex slices ($F(3,23) = 0.2354$; $P = 0.8706$) (Fig. 1). LGA (5 mM) had no effect on this parameter either in slices ($t(7) = 0.45$; $P = 0.671$) or in homogenates ($t(7) = 0.849$; $P = 0.424$) (results not shown).

Fig. 2 shows the in vitro CO_2 production from [U-^{14}C]acetate in rat cerebral cortex homogenates in the presence of 1.0, 2.5 or 5.0 mM DGA (Fig. 2A) or LGA (Fig. 2B). Values are expressed as percentage of controls. CO_2 production ranged from 81 to 156 μmol h^{-1} (mg tissue)$^{-1}$ in the control group. It can be seen that 5 mM DGA significantly reduced CO_2 production in homogenates ($F(3,27) = 3.1537$; $P = 0.0433$), whereas LGA had no effect ($F(3,15) = 0.6739$; $P = 0.5845$). Fig. 3 shows the in vitro CO_2 production from [U-^{14}C]acetate in rat cerebral cortex slices in the presence of 5.0 mM DGA (Fig. 3A) or LGA (Fig. 3B). DGA significantly reduced CO_2 production, whereas LGA had no effect ($F(2,11) = 4.4614$; $P = 0.0451$).

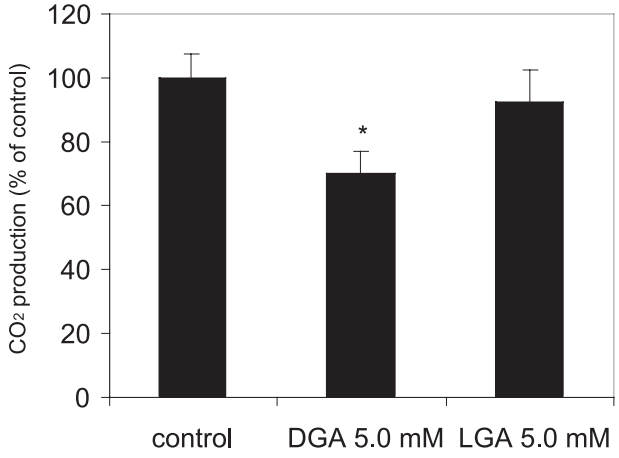

Fig. 3. Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on in vitro CO2 production from [U-^{14}C]acetate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex slices of 30-day-old rats. Values are mean ± S.E.M. for $n = 4$ per group, and are expressed as percentage of controls. *$P < 0.05$, compared to controls (Duncan multiple range test).

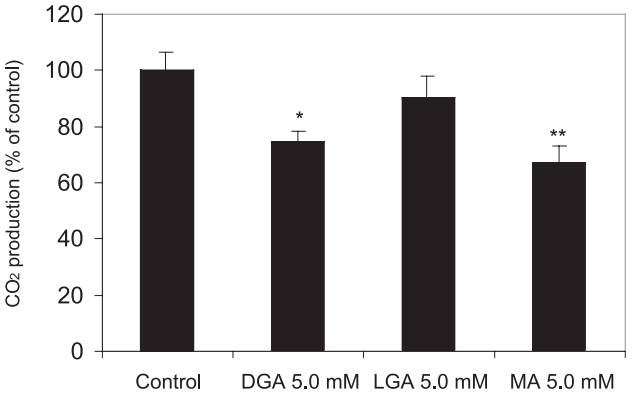

Fig. 4. Effect of D-2-hydroxyglutaric acid (DGA), L-2-hydroxyglutaric acid (LGA) and malonate (MA) on in vitro CO2 production from [U-^{14}C]citrate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex slices of 30-day-old rats. Values are mean ± S.E.M. for $n = 3$ per group, and are expressed as percentage of controls. *$P < 0.05$, compared to controls (Duncan multiple range test).

We also examined the effect of 5 mM LGA and DGA on the in vitro CO_2 production from [U^{14}C]citrate by cerebral cortex slices (Fig. 4). In these experiments we also used 5 mM malonate (MA), a classical and potent succinate dehydrogenase inhibitor. The results showed that malonate and DGA significantly inhibited CO_2 production, whereas LGA caused no effect ($F(3,11) = 8.1022$; $P = 0.0083$).

Next, we investigated the effect of DGA and LGA on the activities of the respiratory chain enzyme complexes in homogenates of rat brain cortex. DGA strongly reduced the activity of cytochrome *c* oxidase (COX) ($F(7,43) = 51.1114$; $P = 0.00001$) in a dose-dependent way, whereas the activities of complex I+III ($F(3,15) = 0.2127$; $P = 0.8856$), complex II ($F(3,23) = 0.1130$; $P = 0.9392$), succinate dehydrogenase (SDH) ($F(3,23) = 0.3832$; $P = 0.7662$), complex II+III ($F(3,15) = 2.4241$; $P = 0.1163$), and complex III ($F(2,23) = 0.9773$; $P = 0.4231$) were not affected by the acid (Fig. 5). In contrast, LGA did not alter these activities (complex I+III: $F(3,15) = 0.04435$; $P = 0.7262$; complex II: $F(3,23) = 0.2242$, $P = 0.8784$; SDH: $F(3,31) = 0.499$, $P = 0.985$; complex II+III, $F(3,15) = 0.1529$; $P = 0.9258$; complex III $F(3,15) = 0.3385$; $P = 0.7999$; complex IV: $F(3,15) = 0.190$; $P = 0.9962$) (results not shown).

The concentration of DGA required to inhibit 50%

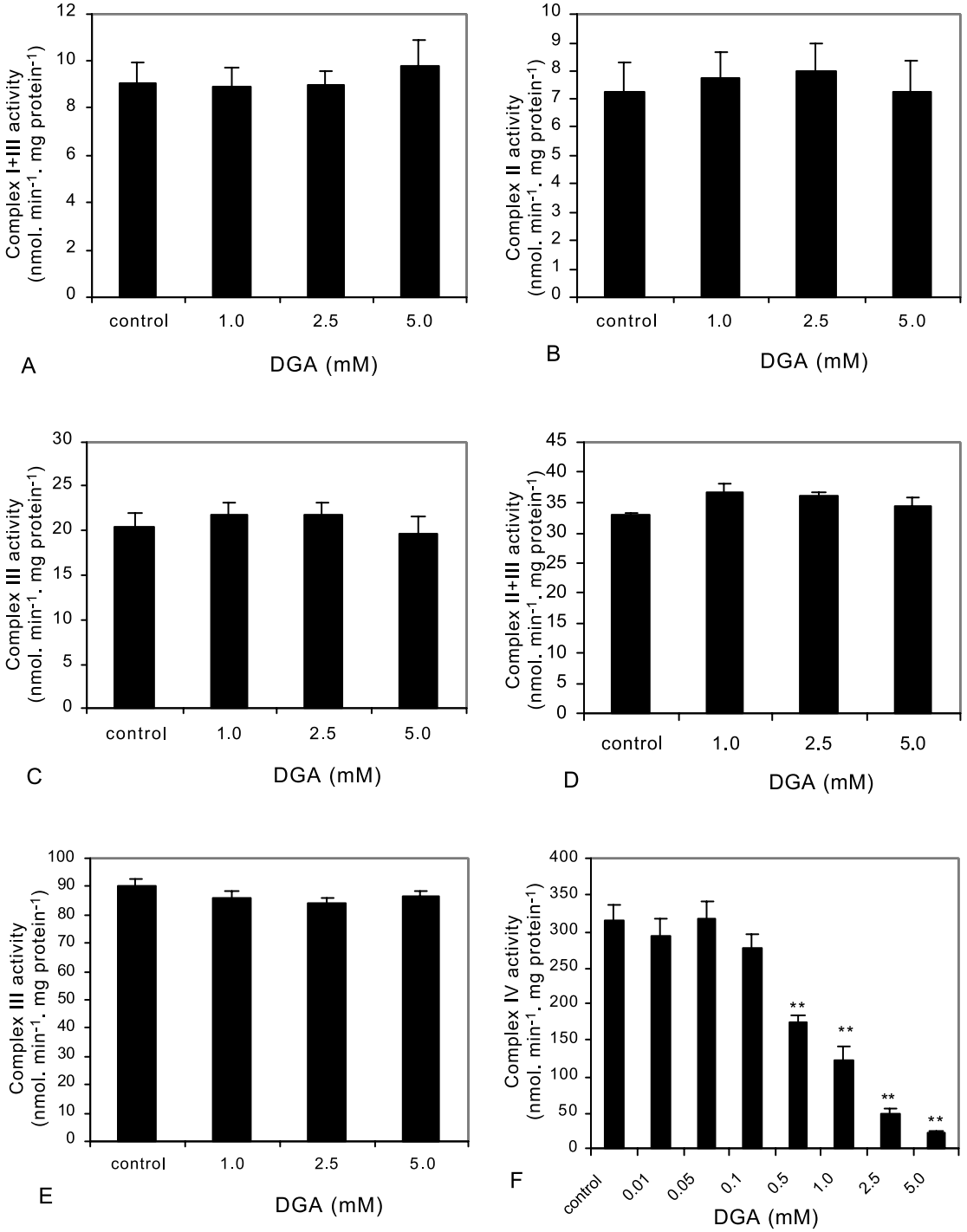

Fig. 5. Effect of D-2-hydroxyglutaric acid (DGA) on the activities (nmol min^{-1} (mg protein)$^{-1}$) of the respiratory chain enzyme complexes in cerebral cortex homogenates of 30-day-old rats. Values are mean ± S.E.M. for $n = 4$–9 independent experiments per group of the respiratory chain complexes activities measured in cerebral cortex homogenates in the presence or absence of the metabolites. $^{**}P < 0.01$ compared to controls (Duncan multiple range test).

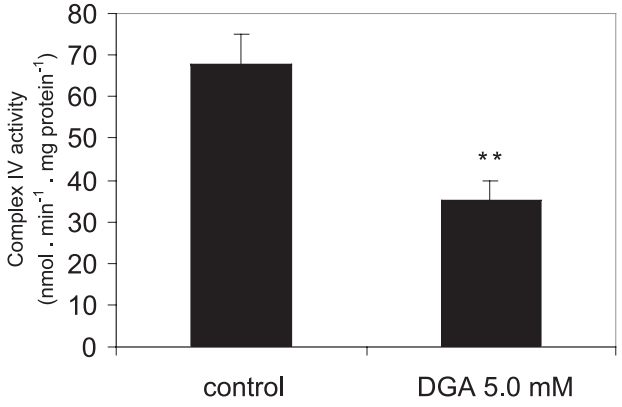

Fig. 6. The effect of D-2-hydroxyglutaric acid (DGA) on cytochrome *c* oxidase activity (nmol min^{-1} (mg protein)$^{-1}$) from human skeletal muscle. Values are mean ± S.E.M. for $n = 6$ per group. $^{**}P < 0.01$ compared to controls (Student *t*-test).

of COX activity in cerebral cortex of rats was measured as the IC_{50} [21]. The data indicated an $IC_{50} = 0.314 \pm 0.061$ mM (mean ± S.D.).

In order to test whether the significant reduction of COX activity caused by DGA was due to a non-specific effect of D-enantiomers, we also tested the effect of 5 mM D-2-hydroxy-3-methylvaleric acid (HMVA), a compound with a molecular mass similar to that of DGA on this activity. The acid did not alter COX activity in homogenates of rat cerebral cortex ($t(6) = 1.09$; $P = 0.319$) (results not shown).

The next step was to investigate whether the inhibition of COX activity verified in rat brain also occurs in human tissues. Thus, the effect of 5 mM DGA on COX activity from human skeletal muscle homogenates was examined. We observed that the acid significantly inhibited COX activity in skeletal muscle ($t(10) = 3.786$; $P = 0.004$) (Fig. 6).

The kinetics of the interaction of DGA with COX in homogenates from cerebral cortex was also determined [22]. The Lineweaver–Burk double-reciprocal plot was analysed over a range of cytochrome *c* concentrations (0.008–0.05 μM) in the absence or presence of DGA (0.5–2.5 mM). The data indicate that the inhibition of COX activity by DGA is uncompetitive (Fig. 7). The K_m calculated was 0.0539 ± 0.011 mM (mean ± S.D., $n = 3$). The K_i value (the dissociation constant of the enzyme–substrate–inhibitor complex) was calculated by the method of Dixon [21], which provides a simple way of determining the inhibition constant (K_i) for uncompetitive inhibitors. The K_i value calculated was 0.226 ± 0.022 mM for DGA (mean ± S.D., $n = 5$).

4. Discussion

High amounts of DGA and LGA accumulate in DHGA and LHGA, respectively. Although severe neurological symptoms and structural brain abnormalities are frequently found in these neurometabolic diseases, very little is known about the pathophysio-

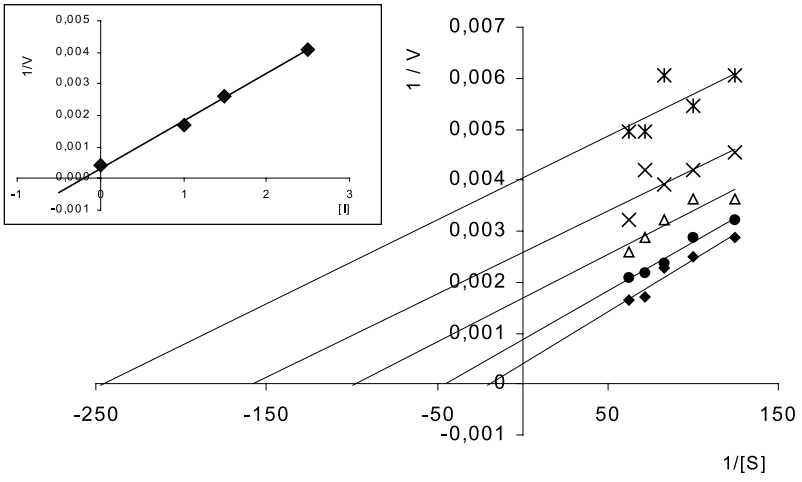

Fig. 7. Kinetic analysis of the inhibition of cytochrome *c* oxidase from cerebral cortex of rats by D-2-hydroxyglutaric acid. The graph shows a double reciprocal plot of cytochrome *c* oxidase for cytochrome *c* concentrations (0.008–0.025 μM) ([S]) in the absence (◆, controls) and in the presence of 0.5 (●), 1.0 (△), 1.5 (×) and 2.5 (*) mM D-2-hydroxyglutaric acid ([I]). The inset shows the Dixon plot for K_i determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

logical mechanisms underlying the neurological dysfunction of DHGA and LHGA [5,23]. The understanding of the biochemical alterations in brain may possibly contribute to a better therapeutic management and consequently to higher survival rates for the affected patients.

The present study was undertaken to evaluate the influence of high concentrations of DGA and LGA on some biochemical parameters of energy metabolism in rat brain. We firstly observed that DGA decreased brain glucose utilization in cerebral cortex homogenates, but not in cortical slices, and that LGA had no effect on this parameter. The lower rate of glucose utilization by homogenates may reflect a decreased Krebs cycle activity. Moreover, the lack of effect of DGA with slices may be due to a lower penetration of the metabolite to all of the cells in the slice. In this context, the next set of experiments were designed to investigate the activity of the Krebs cycle by measuring CO_2 production from acetate. A significant reduction of CO_2 production in brain homogenates and slices was detected due to the presence of DGA in the incubation medium, whereas LGA had no effect on this parameter. Although we do not know how DGA crosses cell membranes, a possible competition between DGA and acetate through the same membrane transporter (monocarboxylic carrier) may reduce CO_2 production from [U^{14}C]acetate. However, this is unlikely since CO_2 generation from citrate, which uses the tricarboxylic carrier to enter mitochondria, was also blocked by DGA. Thus, the data are indicative that the inhibitory action of DGA is probably secondary to a blockage of the Krebs cycle and/or the respiratory chain.

In this context, we verified that DGA strongly inhibited COX (complex IV) activity in a concentration-dependent manner, whereas the other activities of the respiratory chain were not changed by the metabolite. On the other hand, LGA did not alter the respiratory chain complex activities studied. The results indicate that DGA is an inhibitor of only one enzyme of the electron transfer respiratory chain involved in the oxidative phosphorylation and responsible for most energy produced in the cell. The kinetic analysis of the effects of DGA on COX activity indicated an uncompetitive inhibition. The K_i value obtained for the inhibition of COX by DGA was of the millimolar order. Therefore, it is likely that suppression of COX activity by DGA is the primary cause of the reduction of both glucose uptake and CO_2 production (Krebs cycle) observed in the present study. Further studies are clearly required to verify the in vivo effect of DGA on COX activity and this investigation is currently in progress in our laboratory.

Furthermore, the observations that LGA did not interfere with the parameters studied and that D-2-hydroxymethylvaleric acid, a D-isomer of molecular mass similar to that of DGA, caused no alteration of COX activity point to a specific effect of DGA, rather than a nonspecific action due to acidic or to D-enantiomer compounds. As regards LGA, our results are in agreement with a previous report demonstrating that the activities of complexes I to IV of the respiratory chain are normal in liver and muscle biopsies from patients with LHGA, indicating that the sustained tissue elevation of LGA does not provoke inhibition of these activities [4].

We also verified in the present study that DGA inhibits COX activities in human skeletal muscle homogenates, and this is not surprising since, due to its critical importance for life support, in general the chemical structure of cytochrome oxidase was well preserved along evolution.

Cytochrome *c* oxidase (COX; EC 1.9.3.1) or complex IV, the last component of the respiratory chain, catalyses the transfer of electrons from cytochrome *c* to molecular oxygen. It consists of 13 subunits, 10 of which are encoded by nuclear DNA [24]. A defect in this complex causes an inability to produce energy aerobically and results in the accumulation of lactic acid. The tissues that are more dependent on aerobic metabolism, such as brain, muscle and heart, are more likely to be affected in these disorders. This is in line with the fact that COX deficiency usually causes an encephalopathy or a myopathy, although the most common clinical presentation is encephalopathy [25–33].

The regulation of the rate of respiration and ATP synthesis was previously thought to be only due to the electrochemical proton gradient across the inner mitochondrial membrane according to the chemiosmotic hypothesis [34]. More recently, another mechanism of respiratory control was attributed to the allosteric inhibition of COX at high intramitochon-

drial ATP/ADP ratios [35–37]. In a recent report, Kadenbach and colleagues [38] demonstrated a reversible and cAMP-dependent phosphorylation of nuclear-coded subunits of COX at high ATP levels causing its inhibition and hypothesized that this may represent a general mechanism of energy metabolism control based on variable efficiency of energy transduction in COX and on the turning on and off of respiratory control via the intramitochondrial ATP/ADP ratio.

Although neurological symptoms are common in primary mitochondrial disorders, very little is known about the influence of the metabolites accumulating in neurodegenerative disorders on the activities of the respiratory chain complexes. In the present study we demonstrated that D-hydroxyglutaric acid strongly inhibits COX activity in brain of young animals. The degree of this inhibition ranged from 45% to 90% according to the concentration of DGA used in the assays (0.5–5 mM). The question which must be raised is whether this extent of inhibition could compromise energy production. A recent report showed that the control of COX flux is tightly regulated in human skeletal muscle in vivo with important implications for mitochondrial myopathies [39]. These investigators demonstrated that significant reductions of the respiratory fluxes are evident even at a low degree of inhibition of the isolated COX. They observed significant effects on the flux control coefficient and the COX reserve capacity in samples having 10–50% deleted mtDNA, reflected by a decline in COX activity and heme aa3 content, and presumed that these findings might possibly explain the pathological phenotype occurring in individuals carrying a low proportion of mutant mtDNA in susceptible tissues. All this indicates a rate limitation of oxidative phosphorylation by COX. Therefore, the degree of inhibition (45–90%) found in the present study probably leads to blockage of the aerobic glycolytic pathway and to energy deprivation. Therefore, it is tempting to speculate that energy deprivation may be related to the symptomatology and brain damage seen in patients affected by DHGA.

In the present study, significant inhibition of COX activity was achieved with 0.5 mM DGA, which is within the blood circulating (46–757 μmol l^{-1}) levels of DGA in patients affected by DHGA [5,6]. Although the brain concentrations of DGA in these patients are yet unknown, we cannot exclude that higher intracerebral concentrations of DGA may be attained in this neurometabolic disease, as possibly occurs in other organic acidemias so-called 'cerebral' organic acidemias whose symptoms are predominantly or almost exclusively neurologic [40]. Therefore, if these results can be extrapolated to the human condition, it is conceivable that this concentration (0.5 mM) may affect the respiratory chain flux in tissues of these patients.

The molecular defect of DHGA has not been established yet. It has been proposed that D-2-hydroxyglutaric aciduria could be due to a primary mitochondrial defect, with a separate ETF-linked of ETF-ubiquinone oxireductase-linked dehydrogenase being the molecular underlying defect [5]. Although tissue accumulation of DGA is the biochemical hallmark of D-hydroxyglutaric aciduria, some of the documented patients excrete increased amounts of lactate and citric acid cycle intermediates or dicarboxylic acids, pointing to a primary or functional mitochondrial dysfunction [6]. The present findings point to a secondary mitochondrial respiratory chain dysfunction caused by the metabolite which most accumulates in this disorder. In this context, the elevation in the levels of Krebs cycle intermediates found in some of the affected patients may occur secondarily to the blockage of the respiratory chain electron flux leading to an increase of NADH and FADH2 concentrations. Increase of these reduced nucleotides may possibly lead to inhibition of alpha-ketoglutaric acid and succinate dehydrogenases and consequently of their precursors alpha-ketoglutarate and succinate. Lactate may also be increased because of the high NADH/NAD^+ ratio. In this context, it would be important to determine whether the DGA levels are more elevated in patients excreting or accumulating these intermediates.

Now concerning to the neuronal degeneration in DHGA, lack of energy might compromise the synthesis of neurotransmitters (acetylcholine, glutamate, aspartate and GABA) from citric acid intermediates [41] and lipid synthesis in brain which could cause serious neurological damage. This may be the case for DHGA where the patients have demyelination and cerebral cortical atrophy [5].

On the other hand, our present findings of impaired mitochondrial energy production in brain

caused by DGA may lead to activation of the NMDA receptors, even at normal concentrations of glutamate in the synaptic cleft, by a reduction of the resting membrane potential, inducing a release of the voltage-dependent Mg^{2+} block of the channel resulting in calcium influx and eventually cell death (slow-onset excitotoxicity) [42]. It should also be stressed that lack of energy may cause a deficient glutamate uptake by glial cells and by neurons, leaving more neurotransmitter in the cleft.

In conclusion, although the biochemical defect of DHGA is still unknown, it is possible that a reduction of COX activity caused by DGA as found in the present study may be related to the neurodegeneration of patients affected by DHGA. Inhibition of this activity could also explain the elevation of some of the Krebs cycle intermediates and lactate in the body fluids of some of these patients. It is also interesting to point out that hypotonia and myocardiopathy, commonly seen in COX deficiency, are frequently observed in DHGA patients with the early severe form. On this basis, it is possible that some of our results may be relevant to the understanding of the biochemical alterations leading to neurologic deterioration in these patients.

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